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Splenic B cells are required for tolerogenic antigen presentation in the induction of anterior chamber-associated immune deviation (ACAID)

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SUMMARY

Ocular immune privilege is the result of a number of protective mechanisms, including a specialized immune response to antigen encountered in the anterior chamber of the eye. Anterior chamber-associated immune deviation, or ACAID, is characterized by the antigen-specific, selective down-regulation of systemic cell-mediated and humoral immune responses. One current hypothesis of the initiation of ACAID predicts that ocular APC process antigen and then migrate out of the eye and to the spleen where various regulatory T-cell populations are generated. A novel *in vitro* model of the ACAID spleen was developed to study the cells involved in the generation of suppressed T-cell immunity. ACAID APC co-cultured with whole splenocytes or splenic B and T cells induced efferent suppressors of delayed-type hypersensitivity (DTH). However, ACAID APC co-cultured with splenic T cells did not generate efferent suppressors of DTH. The requirement for B cells was confirmed with B-cell knockout mice. ACAID APC co-cultured with splenocytes from B-cell knockout mice did not induce efferent suppressors of DTH. Moreover, ACAID could not be induced in B-cell knockout mice *in vivo*. The reconstitution of B-cell knockout mice with wild-type B cells restored ACAID. In summary, these data confirm the role for B cells in the splenic phase of ACAID. A putative mechanism predicts that ACAID APC release antigenic peptides to B cells in the spleen. B cells then present antigen in a tolerogenic manner leading to the generation of regulatory T cells.

INTRODUCTION

Immune privilege in the anterior chamber of the eye permits histoincompatible tissues and grafts to enjoy prolonged, and sometimes permanent, survival as compared with other anatomical sites.[1] Ocular immune privilege is not a singular event, but rather is the sum total of a number of protective mechanisms including:

(1) the absence of lymph vessels draining the interior of the eye;[2]

(2) the presence of multiple immunosuppressive factors in the aqueous humor;[3]

(3) the extensive expression of Fas-ligand;[4] and

(4) a protective immune response to antigen encountered in the anterior chamber of the eye, or anterior chamber-associated immune deviation (ACAID).[5]

ACAID is characterized by the antigen-specific down-regulation of systemic delayed-type hypersensitivity (DTH) and complement-fixing antibody isotypes.[6]

Several investigators have demonstrated that an intact spleen is necessary for the induction of ACAID, regardless of the nature of the antigen introduced into the anterior chamber.[5,7–9] *In vivo* studies, which compared the induction of ACAID with either soluble or particulate antigens, revealed that soluble antigens introduced into the anterior chamber induce the generation of a blood-borne, cell-associated signal that arises in the eye and is transduced in the spleen.[10,11] Moreover, within 24–48 hr of anterior-chamber priming with bovine serum albumin, the blood-borne cellular signal could be detected in the form of F4/80$^+$ mononuclear cells.[12] The F4/80$^+$ antigen-presenting cells (APC), induced by anterior-chamber priming with antigen, preferentially migrate to the spleen where they deliver an antigen-specific signal that leads to the down-regulation of DTH.[12,13] It has been proposed that these cells originate in the eye where they capture intraocular antigen before migrating to the spleen and inducing T cells which down-regulate DTH.[14] Similar down-regulatory APC can be generated *in vitro* by pulsing F4/80$^+$ macrophages with antigen in the presence of aqueous humor or supernates of

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Abbreviations: ACAID, anterior chamber-associated immune deviation; AC, anterior chamber; APC, antigen-presenting cell(s); DTH, delayed-type hypersensitivity; LAT, local adoptive transfer; PEC, peritoneal exudate cells; Th2, T helper 2.

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iris/ciliary body cell cultures. Although aqueous humor contains a potpourri of immunosuppressive cytokines, transforming growth factor-β (TGF-β) appears to be the major aqueous humor factor that affects APC.[14] Non-ocular APC exposed to TGF-β preferentially accumulate in the spleen following intravenous injection and induce antigen-specific down-regulation of DTH, which is indistinguishable from the ACAID phenotype.[13,15,16] Moreover, the capacity of supernates from iris/ciliary body cell cultures to generate suppressive APC *in vitro* can be abrogated by the addition of neutralizing anti-TGF-β antibody.[14] Thus, exposing non-ocular F4/80$^+$ macrophages to the ocular cytokine, TGF-β, promotes the development of down-regulatory APC, which behave in a manner analogous, if not identical, to the blood-borne F4/80$^+$ mononuclear cells induced by anterior-chamber priming.

Although there is much evidence that the spleen is absolutely required for the development of ACAID, the exact cells and mechanisms involved in the generation of the suppressor T-cell populations remain a mystery. Previous major histocompatibility complex (MHC)-restriction studies have suggested that ACAID APC may present antigen directly to splenic T cells.[17] Subsequent studies with anti-μ-treated mice have shown that the splenic B cell is a necessary component of ACAID and have suggested that the splenic B cell is involved in antigen presentation.[18] Recent studies have shown that the ACAID state correlates with a T-helper 2 (Th2)-like response in which splenic T cells produce interleukin-10 (IL-10), but not interleukin-2 (IL-2) or interferon-γ (IFN-γ) *in vitro*.[19–21] As B cells characteristically elicit a Th2 response,[22,23] these data support the hypothesis that B cells are involved in ACAID-inducing antigen presentation.

An *in vitro* model of the ACAID spleen was developed to study the role of B cells in ACAID. A previously described *in vitro* model of the anterior chamber was used to mimic the ocular component of ACAID.[14] Because ocular APC are thought to migrate out of the eye and preferentially home to the spleen, ACAID APC were co-cultured with whole splenocytes *in vitro*. Once established, it was determined whether ACAID APC could present antigen directly to splenic T cells, or whether the splenic B cell was a necessary participant in the generation of ACAID regulatory T cells. It was hypothesized that ACAID could not be induced in mice that were genetically deficient in mature, functional B cells and that the reconstitution of normal B cells would restore ACAID.

MATERIALS AND METHODS

Mice

C57BL/6 and BALB/c mice (6–10 weeks of age) were obtained from the mouse colony at the University of Texas Southwestern Medical Center (Dallas, TX). C57BL/6 B-cell-deficient mice (C57BL/6-Igh-6^{tm1Cgn}) and BALB/c *nu/nu* mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Preparation of peritoneal exudate cells

Peritoneal exudate cells (PEC) were collected by peritoneal lavage of mice that had been injected intraperitoneally (i.p.) with 1·5 ml of 3% thioglycolate medium (Sigma Chemical Co., St. Louis, MO) 3–4 days previously.

In vitro *model of the anterior chamber (eye-in-a-dish)*

In a previously described model of the anterior chamber of the eye, TGF-β-treated PEC were found to mimic the function of ocular ACAID APC.[14] PEC from normal mice were collected and suspended in complete RPMI-1640 (JRH Biosciences, Lenexa, KS) containing 10% fetal bovine serum (FBS) (HyClone Laboratories, Inc., Logan, UT), 2 mM L-glutamine (JRH Biosciences), 10 mM HEPES buffer solution (JRH Biosciences), 1 mM sodium pyruvate solution (JRH Biosciences), 1% non-essential amino acids solution (BioWhittaker, Walkersville, MD) and 1% penicillin–streptomycin–fungizone solution (BioWhittaker). Plastic-adherent PEC were collected by incubation on plastic tissue culture dishes (Falcon 3803, Becton Dickinson Labware, Lincoln Park, NJ) at 37° in 5% CO_2 for 2·5 hr. Non-adherent cells were washed off with three washes in Hanks' balanced salt solution (HBSS) and plastic-adherent PEC were collected by gentle dislodging with a Nitex filter swab (Tetko, Briarcliff Manor, NY). The cells were then resuspended in complete RPMI (cRPMI) and aliquoted at $1{\cdot}5 \times 10^6$ cells/well in a 24-well plastic tissue culture plate (Falcon 3047, Becton Dickinson Labware) with 5 mg/ml ovalbumin (OVA; Sigma Chemical Co.), with or without 2 ng/ml recombinant human TGF-β_2 (Genzyme Diagnostics, Cambridge, MA). Cell cultures were incubated at 37° in 5% CO_2 overnight. All cells were collected and thoroughly washed twice (30–50 ml HBSS). The majority (90%) of these cells have been shown to express the monocyte/macrophage marker F4/80.[21] Flow cytometry indicates that these cells express the macrophage marker Mac-1 (95%), but few express the dendritic-cell markers CD11c (5%) or DEC-205 (13%) (data not shown). The role of macrophages was confirmed in experiments in which TGF-β-treated PEC (ACAID APC) were treated *in vitro* with anti-Mac-1 antibody prior to functional analysis. Anti-Mac-1-treated ACAID APC were incapable of inducing suppressed DTH (data not shown). By contrast, treatment of ACAID APC with a cocktail of anti-CD11c and anti-DEC-205 antibodies did not affect the suppression of DTH (data not shown). Therefore, the ACAID-inducing population of cells are Mac-1$^+$, CD11c$^-$, DEC-205$^-$. After overnight culture, the ACAID APC were either infused intravenously ($2–5 \times 10^5$ cells in 150 μl) into naive, syngeneic recipients or placed into culture with various splenocyte components.

In vitro *model of the spleen (spleen-in-a-dish)*

TGF-β-treated, adherent APC ($1–3 \times 10^6$ cells) in cRPMI were placed into large petri dishes (Falcon 3003; Becton Dickinson Labware). Syngeneic splenocytes were harvested and erythrocytes lysed. In some experiments, $50–100 \times 10^6$ whole BALB/c splenocytes (approximately one spleen) were added to the cultures. In other experiments, B-cell knockout splenocytes ($12{\cdot}5 \times 10^6$ cells) or B6 splenocytes ($12{\cdot}5 \times 10^6$ cells) were added. In others, splenic B and T cells were isolated by incubating spleen cells on plastic tissue culture dishes, as described above. Non-adherent B and T cells were washed off and collected in HBSS. One spleen equivalent of B and T cells ($55–70 \times 10^6$ cells in cRPMI) was added to the cultures. In other experiments, splenic T cells were isolated by incubating the whole spleen cell suspension on scrubbed nylon wool (Fenwal Laboratories, Deerfield, IL) at 37° in 5% CO_2 for 1 hr. T cells were eluted with 30 ml of HBSS. One spleen

equivalent of T cells ($30–45 \times 10^6$ cells in cRPMI) was added to the cultures. In other experiments, BALB/c *nu/nu* splenocytes (90×10^6 cells) were added. In all spleen-in-a-dish experiments, 20 μg OVA was added to simulate antigen escape from the eye *in vivo*. Complete RPMI was added to a total volume of 10–15 ml. All dishes were incubated at 37° in 5% CO_2 for 5–7 days with frequent agitation. The generation of T cells capable of suppressing the expression of DTH was tested by local adoptive transfer assay.

Local adoptive transfer (LAT) assay

A LAT assay was performed as previously described.[24] Briefly, primed effector T cells were obtained from mice immunized subcutaneously (s.c.) 7–14 days earlier with 250 μg OVA emulsified 1:1 in complete Freund's adjuvant (CFA) (0·5 mg *Mycobacterium*/ml, Behring Diagnostics, La Jolla, CA). T cells were resuspended at $5–10 \times 10^7$ cells/ml in 10 mg/ml OVA in sterile phosphate-buffered saline (PBS). Non-adherent spleen-in-a-dish culture cells were collected and resuspended at $5–10 \times 10^7$ cells/ml in 10 mg/ml OVA. Equivalent numbers of primed T cells and test cells were mixed. Positive controls contained primed T cells mixed with naive splenocytes. Negative controls contained naive splenocytes alone. Cell suspensions (20 μl = 2×10^6 cells) were injected into left ear pinnae of naive, syngeneic mice. The right ear pinnae received 20 μl of 10 mg/ml OVA alone. Ear swelling was measured at 24 hr.

Reconstitution of B-cell-deficient mice

C57BL/6 wild-type spleen cells were harvested and erythrocytes lysed. B cells were isolated by panning on plastic dishes coated with goat anti-mouse gamma globulin (ICN Pharmaceuticals, Inc., Aurora, OH), as previously described.[18] The purified B cells were resuspended to 5×10^7 cells/ml in cRPMI, filtered through sterile Nitex (Tetko, Inc.) and injected (10^7 B cells) intravenously into B-cell-deficient mice. Successful reconstitution was assayed four (Day 0) and 11 (Day 7) days later by flow cytometry.

Intracameral inoculation

Mice were anaesthetized with 0·66 mg ketamine hydrochloride (Vetalar; Park-Davis and Co., Detroit, MI) given i.p. A glass micropipette (approximately 80 μm diameter) was fitted onto a sterile infant-feeding tube (no. 5 French; Professional Medical Products Inc., Greenwood, SC) and mounted onto a 0·1 ml Hamilton syringe (Hamilton Co., Inc., Whittier, CA). A Hamilton automatic dispensing apparatus was used to dispense 5 μl of a 20 mg/ml OVA (Sigma Chemical Co.) solution in sterile PBS (= 100 μg OVA) into the anterior chamber via the glass micropipette.

Subcutaneous inoculations

Mice were immunized by s.c. injection of OVA (125–250 μg), emulsified 1:1 in CFA, in a total volume of 100 μl.

Delayed-type hypersensitivity (DTH) assay

Both ear pinnae of experimental and control animals were measured with a Mitutoyo engineer's micrometer immediately prior to challenge. OVA (400 μg) in 20 μl PBS was injected s.c. into the left ear pinnae. The right ear pinnae received 20 μl sterile PBS alone (negative control). Both ear pinnae were measured 24 hr later and the difference in ear pinnae size was used as a measure of DTH. Results were expressed as: specific ear pinnae swelling = (24-hr measurement − 0-hr measurement) for experimental ear − (24-hr measurement − 0-hr measurement) for control ear.

Statistics

All experimental and control groups contained five animals ($n = 5$). Differences between groups were analysed by the Student's *t*-test. *P*-values $< 0·05$ were considered significant.

RESULTS

The *in vitro* generation of ACAID regulatory T cells

An *in vitro* model of the ACAID spleen (spleen-in-a-dish) was developed to dissect the spleen cells involved in the induction of ACAID. Upon encountering antigen in the eye, ocular APC are thought to migrate out of the eye and home preferentially to the spleen.[13] Accordingly, ACAID APC were co-cultured with whole splenocytes *in vitro*. The generation of T cells capable of suppressing the expression of DTH (i.e. efferent suppressors) was used as a characteristic measure of ACAID.

ACAID APC were generated using a previously described *in vitro* model of the eye.[14] Peritoneal macrophages incubated in the presence of aqueous humor or the ocular cytokine, TGF-β, subsequently function as ocular APC when injected *in vivo*.[14,25] Briefly, plastic-adherent peritoneal exudate cells were cultured overnight in the presence of 5 mg/ml OVA and in the presence or absence of 2 ng/ml TGF-β. The next day, the cells were collected, thoroughly washed and placed into culture with one spleen equivalent of syngeneic, whole spleen cells. To mimic minimal antigen escape from the eye, 20 μg OVA were added to the cultures. Seven days later, non-adherent cells were collected and tested for the presence of ACAID regulatory T cells by a local adoptive transfer (LAT) assay. Briefly, the non-adherent population of *in vitro*-generated regulatory cells was mixed with OVA and effector T cells that were generated in mice immunized previously. The cells were then injected s.c. into the ear pinnae of naive mice and ear swelling was measured as a gauge of DTH. A reduction in ear swelling, as compared with positive controls, was indicative of regulatory T cells capable of suppressing the expression of DTH. As shown in Fig. 1, ACAID APC co-cultured with whole splenocytes *in vitro* induced T cells that suppressed the expression of DTH. In contrast, regulatory T cells were not generated when OVA-pulsed normal APC were used (Fig. 1). These results were confirmed with another soluble antigen, bovine serum albumin (data not shown). In summary, these data show that at least one characteristic population of ACAID regulatory T cell can be generated in a novel *in vitro* model of the ACAID spleen.

ACAID APC do not present antigen directly to splenic T cells

The hypothesis that ocular APC present antigen directly to splenic T cells was tested by incubating ACAID APC with purified splenic T cells in the spleen-in-a-dish model. ACAID APC were generated by incubating plastic-adherent PEC in the presence of 5 mg/ml OVA and 2 ng/ml TGF-β overnight. The next day, the cells were thoroughly washed and placed into culture with splenic T cells. Seven days later, non-adherent

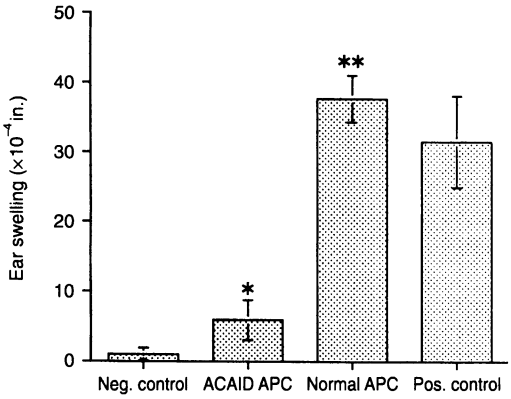

Figure 1. ACAID regulatory T cells can be generated *in vitro*. Adherent BALB/c PEC were incubated overnight with 5 mg/ml OVA and in the presence (ACAID APC) or absence (Normal APC) of 2 ng/ml TGF-β. The next day, all cells were collected, washed and 1×10^6 cells were placed into culture with OVA and one equivalent (5×10^7 cells) of syngeneic spleen cells. Seven days later, non-adherent cells were collected and tested in a LAT assay for the presence of T cells capable of suppressing the expression of DTH. All results are expressed as mean swelling ± SEM. $*P = 0{\cdot}0032$ for ACAID APC versus Positive control (Pos. control). $**P > 0{\cdot}05$ for Normal APC versus Positive control.

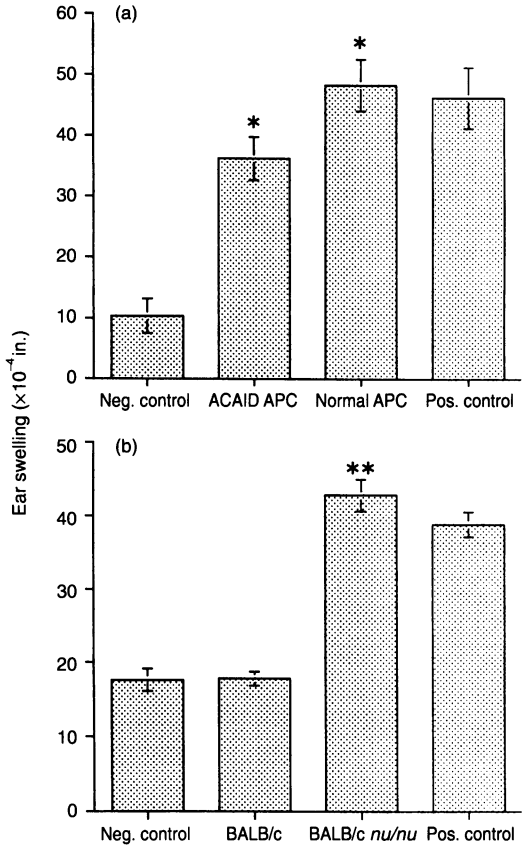

Figure 2. (a) ACAID APC do not present antigen directly to regulatory T cells. BALB/c ACAID APC and normal APC were cultured with OVA and approximately one equivalent of splenic T cells. Seven days later, non-adherent cells were collected and tested in a LAT assay for the presence of T cells capable of suppressing the expression of DTH. (b) T cells are necessary for the *in vitro* generation of regulatory cells. BALB/c ACAID APC were cultured with OVA and whole splenocytes from BALB/c normal (BALB/c) or nude (BALB/c *nu/nu*) mice. Seven days later, non-adherent cells were collected and tested in a LAT assay for the presence of T cells capable of suppressing the expression of DTH. All results are expressed as mean swelling ± SEM. $*P \leq 0{\cdot}0002$ for ACAID APC and Normal APC versus negative control (Neg. control). $**P = 0{\cdot}0001$ for BALB/c *nu/nu* versus Negative control.

cells were collected and tested for regulatory activity in a LAT assay. Both ACAID and normal OVA-pulsed APC were unable to induce efferent suppressors of DTH when co-cultured with purified splenic T cells (Fig. 2a). These results show that ACAID APC do not present antigen directly to splenic T cells such that efferent suppressors are generated. Combined with the previous experiment, these data indicate that a spleen cell not included in the purified T-cell preparation is necessary for the generation of ACAID.

To confirm that T cells are involved in the generation of efferent suppressors of DTH, a spleen-in-a-dish assay was performed using whole splenocytes from BALB/c *nu/nu* mice. Except for the lack of mature, functional T cells, nude mice contain a full complement of all other spleen cell types. Both ACAID and normal OVA-pulsed APC were unable to induce efferent suppressors of DTH when co-cultured with whole splenocytes from nude mice (Fig. 2). In total, these results show that splenic T cells are necessary, but not sufficient, for the generation of efferent suppressors of DTH in an *in vitro* model of the ACAID spleen.

B cells are a necessary component of the ACAID spleen

Previous studies have shown that *in vivo* antibody depletion of B cells prevents the induction of ACAID.[18] It was therefore hypothesized that the generation of efferent suppressors in ACAID required splenic B cells in addition to splenic T cells and ACAID APC. We tested this hypothesis by incubating ACAID APC with purified splenic B and T cells *in vitro*. ACAID and normal OVA-pulsed APC were generated as described above. Splenic B and T cells were purified by incubating whole spleen cell suspensions on plastic dishes. Non-adherent B and T cells were collected and co-cultured with ACAID or normal APC. Seven days later, the generation of efferent suppressors was tested in a LAT assay. As shown in Fig. 3, ACAID APC co-cultured with splenic B and T cells induced the generation of efferent suppressors of DTH. As before, these cells were absent in cultures containing normal OVA-pulsed APC (Fig. 3). In summary, these data show that the generation of efferent suppressors of DTH requires splenic B cells in addition to T cells and ACAID APC.

It was uncertain whether the preparations of purified B and T cells might have contained other spleen-cell types that were involved in the restoration of the ACAID phenotype. To control for the contribution of other spleen-cell types and to confirm the requirement for splenic B cells, we utilized mice that contained a targeted disruption of the immunoglobulin heavy chain gene. These mice specifically lack mature, functional B cells.[26] C57BL/6 (B6) ACAID and normal OVA-pulsed APC were generated as above and placed into culture with whole spleen cells from syngeneic, B-cell knockout mice. Seven days later, the generation of efferent suppressors was

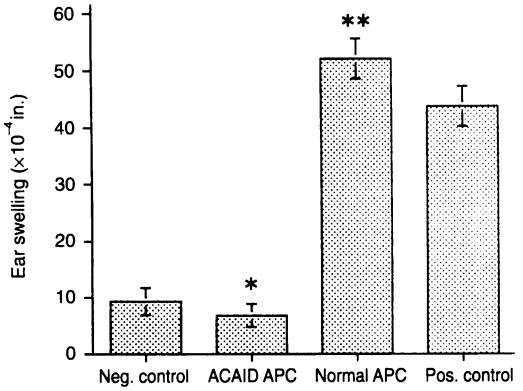

Figure 3. B cells restore ACAID *in vitro*. BALB/c ACAID APC and normal APC were incubated with OVA and one equivalent of splenic B and T cells. Seven days later, non-adherent cells were collected and tested in a LAT assay for the presence of T cells capable of suppressing the expression of DTH. All results are expressed as mean swelling ± SEM. $*P = 0{\cdot}0001$ for ACAID APC versus Positive control. $**P > 0{\cdot}05$ for Normal APC versus Positive control.

tested in a LAT assay. Normal or ACAID APC co-cultured with B-cell knockout splenocytes were unable to generate efferent suppressors of DTH (Fig. 4). Efferent suppressors were induced, however, when ACAID APC were co-cultured with B6 splenocytes (Fig. 4). These *in vitro* data confirm that the B cell is a necessary component for the generation of efferent suppressors in the *in vitro* model of the ACAID spleen.

ACAID cannot be induced in B-cell knockout mice

Results from the *in vitro* experiments indicate that the B cell plays a crucial role in the splenic phase of ACAID. Additional experiments were performed to confirm this hypothesis *in vivo*. OVA (100 μg) was injected into the anterior chambers (AC) of B-cell knockout and normal, syngeneic B6 mice. Seven days later, all mice were immunized s.c. with 125 μg OVA in CFA. Seven days later, DTH was assessed. ACAID was induced when OVA was injected into the AC of normal B6 mice (Fig. 5). In contrast, OVA injected into the AC of B-cell knockout mice did not induce ACAID (Fig. 5). These data confirm that the B cell plays a crucial role in the induction of ACAID *in vivo*.

APC function is intact in B-cell knockout mice

The current model of the initiation of ACAID proposes that tolerogenic APC in the eye process antigen and migrate to the spleen where they are subsequently involved in the generation of regulatory T cells.[11,27] The next series of experiments was designed to rule out the possibility that APC from B-cell knockout mice are incapable of delivering an ACAID-inducing signal *in vivo*. B-cell knockout APC were first tested for the ability to generate ACAID. Briefly, plastic-adherent PEC from normal B6 and B-cell knockout mice were cultured overnight in the presence of 5 mg/ml OVA and 2 ng/ml TGF-β. The next day, the cells were collected, thoroughly washed, and injected i.v. into normal B6 mice. Seven days later, all mice were immunized s.c. with 125 μg OVA in CFA. Seven days later, DTH was assessed. B6 APC incubated with OVA and TGF-β (B6 ACAID APC) generated ACAID in B6 mice (Fig. 6). In addition, B-cell knockout APC incubated with OVA and TGF-β (B-cell k.o. ACAID APC) induced ACAID in B6 mice (Fig. 6). B-cell knockout APC pulsed with antigen in the absence of TGF-β did not induce ACAID (Fig. 6). These results show that APC from B-cell knockout mice are able to generate and deliver an ACAID signal to the spleen.

Because APC from B-cell knockout mice are capable of delivering an ACAID-inducing signal to the spleens of normal mice, the inability of the B-cell knockout mouse to develop ACAID is most likely to be at the level of the spleen. This was tested by generating B6 ACAID APC *in vitro* and testing their capacity to induce ACAID in B-cell knockout mice. Cells

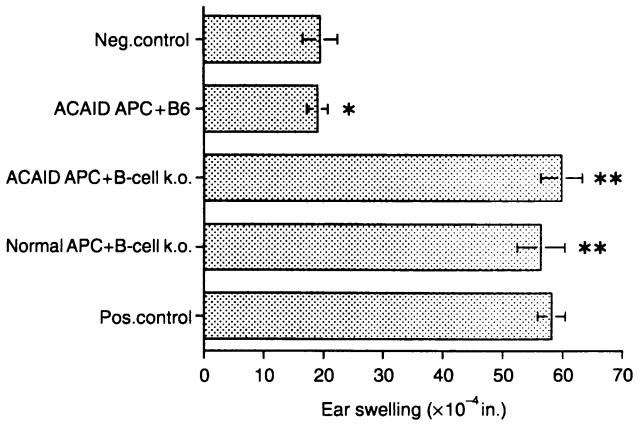

Figure 4. B-cell knockout (k.o.) splenocytes do not support ACAID. B6 ACAID APC and normal APC were cultured with one equivalent of syngeneic, B-cell knockout splenocytes (+B-cell k.o.) or an equivalent number of B6 splenocytes (+B6). Seven days later, non-adherent cells were collected and tested in a LAT assay for the presence of T cells capable of suppressing the expression of DTH. All results are expressed as mean swelling ± SEM. $*P = 0{\cdot}0001$ for ACAID APC +B6 versus Positive control. $**P > 0{\cdot}05$ for ACAID APC +B-cell k.o. and Normal APC+B-cell k.o. versus Positive control.

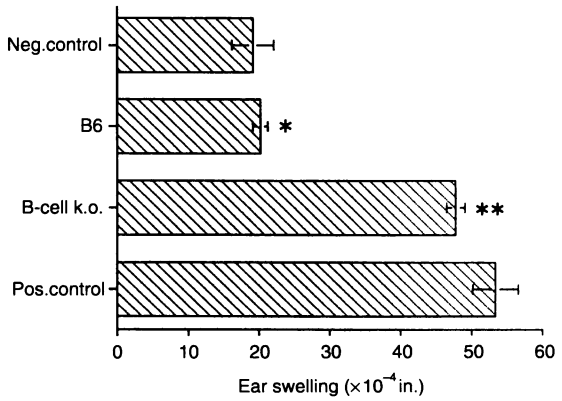

Figure 5. ACAID is not induced in B-cell knockout (k.o.) mice. Soluble OVA (1 μg) was injected into the anterior chambers of normal B6 and B-cell knockout (B-cell k.o.) mice. Seven days later, all mice were immunized s.c. with 125 μg OVA in CFA. DTH to OVA was assessed 7 days later. Positive control animals (Pos. control) were immunized s.c. with 125 μg OVA in CFA. Negative control animals (Neg. control) were not immunized. All results are expressed as mean swelling ± SEM. $*P = 0{\cdot}0001$ for B6 versus Positive control. $**P > 0{\cdot}05$ for B-cell k.o. versus Positive control.

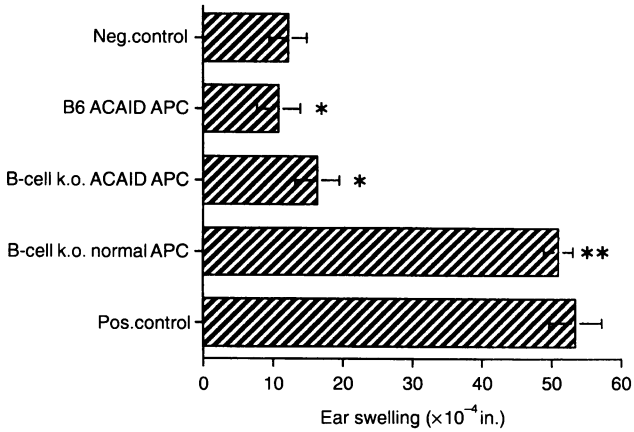

Figure 6. B-cell knockout APC can generate ACAID. Adherent B6 or B-cell knockout (B-cell k.o.) PEC were incubated overnight with 5 mg/ml OVA and in the presence (ACAID APC) or absence (Normal APC) of 2 ng/ml TGF-β. The next day, all cells were collected, washed and 4×10^5 cells injected i.v. into naive B6 mice. Seven days later, all mice were immunized s.c. with 125 μg OVA in CFA. DTH to OVA was assessed 7 days later. Positive control animals (Pos. control) were immunized s.c. with 125 μg OVA in CFA. Negative control animals (Neg. control) were not immunized. All results are expressed as mean swelling ± SEM. *$P = 0{\cdot}0001$ for B6 ACAID APC and B-cell ACAID APC versus Positive control. **$P > 0{\cdot}05$ for B-cell k.o. Normal APC versus Positive control.

were injected i.v. into wild-type B6 or syngeneic B-cell knockout mice. Seven days later, all mice were immunized s.c. with 125 μg OVA in CFA. Seven days later, DTH was assessed. B6 ACAID APC generated ACAID when injected i.v. into normal B6 mice (Fig. 7). ACAID was not induced, however, when B6 ACAID APC were injected i.v. into B-cell knockout mice (Fig. 7). In summary, these results suggest that even though an intact blood-borne ACAID-inducing signal reaches the spleen, it cannot be transduced unless an intact B-cell population is present.

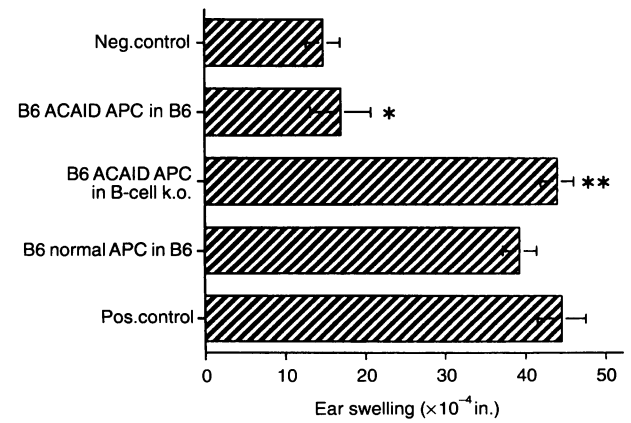

Figure 7. The ACAID lesion resides in the spleens of B-cell knockout mice. ACAID or normal APC from B6 mice were injected i.v. into naive B6 or B-cell knockout (B-cell k.o.) mice. All mice were immunized and tested for DTH as described in Fig. 6. The results are expressed as mean swelling ± SEM. *$P = 0{\cdot}0002$ for B6 ACAID APC in B6 versus Positive control. **$P > 0{\cdot}05$ for B6 ACAID APC in B-cell k.o. versus Positive control.

B cells rescue ACAID in B-cell knockout mice

It was hypothesized that the reconstitution of B cells would restore ACAID in B-cell knockout mice. Briefly, normal B cells were collected by panning on plastic dishes coated with antibody to mouse gamma globulin. B cells (10^7) were injected i.v. into syngeneic B-cell knockout mice. Four and 11 days later, the B-cell marker, B220, was used to detect the presence of B cells in the spleens of reconstituted mice. Fluorescence-activated cell sorter (FACS) analysis measured a significant number of $B220^+$ B cells in the spleens of reconstituted versus naive B-cell knockout mice (approximately 10% above naive background; data not shown). To test the induction of ACAID, 100 μg OVA was injected into the AC of normal B6 mice, naive B-cell knockout mice and B-cell knockout mice 4 days after reconstitution with B cells. OVA injected into the AC of naive B-cell knockout mice did not induce ACAID (Fig. 8). The reconstitution of B cells in B-cell knockout mice, however, restored the generation of ACAID (Fig. 8). These data show that the generation of ACAID is blocked by the absence of splenic B cells in B-cell knockout mice and that the addition of B cells restores the ability of these mice to generate ACAID.

DISCUSSION

The anterior chamber of the eye lacks patent lymphatic drainage channels and it is believed that the primary mode of antigen escape is via the venous route.[8,28,29] Accordingly, delivering antigen into the anterior chamber might be likened to an i.v. injection. As i.v. injection of antigen is known to induce immune deviation and suppression of DTH,[30–35] it has been proposed that the ACAID phenotype is merely a reflection of immune deviation induced by venous egression of antigen from the eye. However, the nature of the down-

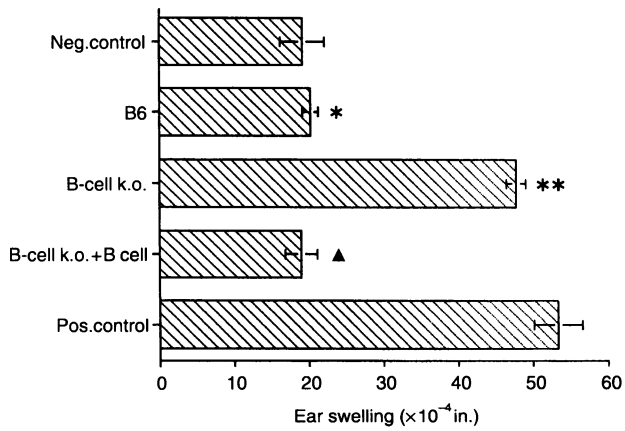

Figure 8. B cells restore ACAID in B-cell knockout mice. B6 splenic B cells were injected i.v. into naive B-cell knockout mice. Four days later, soluble OVA (1 μg) was injected into the anterior chambers of B6, B-cell knockout (B-cell k.o.) and B-cell knockout mice reconstituted with syngeneic B cells (B-cell k.o. + B cell). All mice were immunized and tested for DTH as described in Fig. 5. The experiment testing B-cell knockout mice reconstituted with syngeneic B cells was performed at the same time as Fig. 5. The Fig. 5 control, B6 and B cell k.o. data, are shown again here. All results are expressed as mean swelling ± SEM. *$P = 0{\cdot}0001$ for B6 versus Positive control. **$P > 0{\cdot}05$ for B-cell k.o. versus Positive control. Filled triangle, $P = 0{\cdot}0001$ for B-cell k.o. + B-cell versus Positive control.

regulatory cells induced by i.v. injection differs markedly from those induced by anterior-chamber presentation in several fundamental ways. ACAID regulatory cells expressing the CD8 cell-membrane marker act at the efferent arm of the immune response whereas $CD8^+$, i.v.-induced regulatory cells act at the afferent arm.[24] Afferent ACAID regulatory cells are $CD4^+$. Moreover, presentation of antigen via the anterior chamber down-regulates DTH responses in previously immunized hosts, while i.v. injection of the same antigen fails to desensitize the preimmune host.[36] Although both ACAID and i.v.-induced immune deviation require an intact spleen wherein DTH regulatory cells are generated,[5,7–9,37] antigen introduced into the anterior chamber is modified or processed in the eye before reaching the spleen. A current hypothesis suggests that resident APC in the eye process antigen and migrate from the eye to the spleen where they deliver an antigen-specific signal for the generation of regulatory T cells.[11,27] These T cells are the effector cells of ACAID, regulating both cell-mediated and humoral immune responses.[6] Although there is much evidence for the requirement and involvement of the spleen in the generation of ACAID, the cells and mechanisms responsible for tolerogenic antigen presentation remain a mystery. It was advantageous to develop an *in vitro* model of the ACAID spleen to study both the cells and mechanisms involved in tolerogenic antigen presentation. These primary studies sought to determine which cells were required to generate regulatory T cells in the ACAID spleen.

If the ocular ACAID APC migrates from the eye to the spleen, then it is reasonable to predict that co-culturing ACAID APC with whole splenocytes *in vitro* would duplicate the conditions for the generation of ACAID regulatory T cells. Studies have shown that a 7-day interval is sufficient for the generation of regulatory T cells in the ACAID spleen.[24] Therefore, we tested our hypothesis by generating[14] and co-culturing ACAID APC with whole splenocytes for 7 days *in vitro*. The generation of regulatory T cells capable of suppressing the expression of DTH (i.e. efferent suppressors) was used as a characteristic measure of ACAID. These data show that ACAID APC co-cultured with syngeneic splenocytes *in vitro* duplicates the generation of ACAID suppressors. Although functional, it is important to appreciate the limitations of this new *in vitro* model of the ACAID spleen. More work is necessary to conclusively show that the *in vitro*-generated suppressor cells are identical to those found in ACAID. Other data have shown that, like ACAID *in vivo*, the suppressor activity is contained in the T-cell population (data not shown). However, the phenotype of this cell has not yet been determined. Even with these stipulations, this is the first demonstration of an *in vitro* model in which to study the generation of regulatory T cells. This model was then used to determine which cells are necessary for the generation of regulatory T cells in the ACAID spleen.

MHC-restriction studies have implicated the ocular APC as the proximal cell in the tolerogenic presentation of antigen to splenic T cells.[17] If true, the ACAID APC would be expected to present antigen directly to regulatory T-cell precursors in the spleen. We tested this hypothesis by incubating ACAID APC with purified splenic T cells. Our results show that ACAID APC co-cultured with splenic T cells do not induce efferent suppressors of DTH. These data suggest that either the ACAID APC do not present antigen directly to splenic T cells or that another spleen cell type contributes a necessary component to tolerogenic presentation.

Experiments have demonstrated a central role for the splenic B cell in the induction of ACAID.[18] It was therefore likely that the missing component was the splenic B cell. This hypothesis was tested by adding B cells back to the *in vitro* system. ACAID APC co-cultured with purified splenic B and T cells were able to elicit the generation of efferent suppressors of DTH. The necessity for B cells was also confirmed by incubating ACAID APC with splenocytes derived from B-cell knockout mice. As expected, ACAID was not induced in the absence of B cells. These data confirm that the splenic B cell contributes a necessary component to tolerogenic antigen presentation and that ACAID APC, T cells and B cells are sufficient to generate efferent suppressors *in vitro*. Although this contribution is still unknown, the data do not discount the hypothesis that tolerogenic B cells might present antigen in the ACAID spleen.

The necessity for B cells *in vivo* was confirmed with B-cell knockout mice. ACAID was abrogated in B-cell knockout mice. Because APC function was intact in these animals, the lesion in ACAID was predicted to exist in the spleen. It was hypothesized that the absence of B cells was directly responsible for the block in ACAID. This hypothesis was confirmed by reconstituting B-cell knockout mice with splenic B cells from syngeneic donors. The results show that the reconstitution of B cells restored the induction of ACAID in B-cell knockout mice. These data are consistent with earlier findings in which chronic treatment with anti-μ antibody prevented ACAID in otherwise normal animals.[18]

In summary, the ACAID APC appears unable to present antigen directly to regulatory T-cell precursors in the spleen. Along with ACAID APC and T cells, the splenic B cell is a necessary component for the generation of ACAID, both *in vitro* and *in vivo*. This would imply one or both of two possible roles for B cells in ACAID: B cells provide a necessary cell-associated or soluble factor, such as TGF-β or IL-10[38–40] and/or B cells serve as the tolerogenic APC in the ACAID spleen. This latter hypothesis is supported by the fact that B cells are known to present antigen in a tolerogenic or Th2-inducing manner[22,23,41–43] and that ACAID correlates with a tolerogenic, Th2-type immune response.[19–21] It was recently reported that activated B cells can elicit $CD8^+$ suppressor T cells by presenting peptide in association with the non-classical class I molecule, Qa-1.[44] A $CD8^+$ T cell is also responsible for the efferent suppression of DTH in ACAID.[24] Together, these data suggest that the role of B cells in the ACAID spleen might be to present antigenic peptides delivered by ocular ACAID APC. The tolerogenic presentation of peptide by B cells, perhaps in the context of Qa-1, is then responsible for the generation of regulatory T cells. Studies are underway to address this role for the B cell in the tolerogenic presentation of antigen in the ACAID spleen.

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